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INTERNATIONAL STUDY ON *ARTEMIA*.

NUTRITIONAL EFFECTS IN TOXICITY TESTS:

USE OF DIFFERENT *ARTEMIA* GEOGRAPHICAL STRAINS

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16. ABSTRACT

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INTERNATIONAL STUDY ON *ARTEMIA*[1].

NUTRITIONAL EFFECTS IN TOXICITY TESTS:

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RUNNING HEADLINE:

Nutritional effects in toxicity tests

ABSTRACT

A series of experiments was conducted to determine whether feeding marine organisms different geographical strains of Artemia prior to or during a toxicity test could significantly alter the results of the test. In each experiment, the test organisms were produced from laboratory brood stocks and divided into two groups; one was fed Reference brine shrimp, the other was fed San Pablo Bay brine shrimp. When the groups were then used simultaneously in toxicity tests, significant differences in the test results were obtained. The LC_{50} values for the animals fed Reference Artemia, were always higher than those for animals fed San Pablo Bay Artemia, in certain cases by 2-4 times. Further, some tests were invalidated because of high control mortality in the group fed the San Pablo Bay strain. The findings indicate that the choice of brine shrimp can affect the outcome of a toxicity test, sometimes to the extent that the test is invalid.

INTRODUCTION

One of the most important variables in the conduct of any biological experiment is the nutrition of the test animals used. Poorly-fed animals can be physiologically abnormal and yield biased results in the experiment (NAS, 1978). The importance of nutrition in aquatic toxicology has been demonstrated by Mehrle et al. (1974, 1977), Winner et al. (1977) and Phillips and Buhler (1979). Their experiments proved that the results of toxicity tests can be significantly altered by the use of different diets fed to the experimental organisms.

The U.S. Environmental Protection Agency's Environmental Research Laboratory at Narragansett, Rhode Island, USA (ERLN) conducts toxicological bioassays to provide a scientific data base for the Agency's regulatory programs in ocean disposal of waste and control of toxic substances in the environment. With this need by EPA and the increase in toxicity testing of larval fish and crustaceans by others has come an increased necessity to feed these organisms live brine shrimp (Artemia) nauplii. However, different geographical sources of brine shrimp are known to differ in quality as food for marine organisms (Bookhout and Costlow, 1970; Beck et al., 1980; Johns et al. 1980, 1981; Klein-MacPhee et al., 1980; Beck and Bengtson, 1982), especially with regard to fatty acids (Schauer et al., 1980) and toxicant burdens (Olney et al., 1980). In recognition of the variable quality and to provide a standard diet for test organisms, Reference Artemia cysts (RAC), of known good quality have been made available to the scientific community (Sorgeloos, 1980) to serve as a standard against which other geographical strains can be tested.

The purpose of this study was to demonstrate Artemia diet quality effects on the results of toxicity tests. We designed a series of experiments

to determine differences in LC_{50} values for groups of three species of marine organisms fed different geographical strains of brine shrimp prior to and/or during toxicity tests. This paper describes the results of those experiments and considers the need for standardization of nutrition in aquatic toxicology.

METHODS

The organisms and toxicants used in these experiments were the mysid (Mysidopsis bahia), the Atlantic silverside (Menidia menidia), both tested with cadmium, and the sheepshead minnow (Cyprinodon variegatus), tested with endosulfan. In all cases, offspring were obtained from common parental stocks in the laboratory and immediately separated into two groups, which were then fed either RAC or San Pablo Bay (SPB) Artemia (San Francisco Bay Brand, Lot 1628) (as stage I nauplii) ad libitum.

Culture Methods

Mysids - From a series of stock cultures that had been maintained in the laboratory for more than three years, females carrying young in their brood pouches were removed and placed overnight in hatching chambers. Juveniles which were released from the brood pouches overnight were collected, separated into two groups and placed in 75-l aquaria with undergravel filters and receiving a constant supply of 25 ± 1°C filtered seawater. Each group of mysids was reared in an aquarium for 70 days (3 generations). They were fed nauplii of the appropriate brine shrimp strain. Adult mysids were collected from the aquaria, and, with the exception of females carrying young, were used in a 48-hour toxicity test. Because mysids which had been fed SPB Artemia had ceased reproduction (see Results section), only the culture of mysids on Reference Artemia was continued for 47 more days

(enough time for the production of two more generations). Adult females bearing young were then removed to the hatching chambers for overnight release of a new cohort of juveniles. These juveniles were collected, separated into two groups and used in a 28-day full life-cycle toxicity test. One group was fed Reference Artemia, the other group San Pablo Bay Artemia, throughout the test.

Atlantic silverside - On two occasions, gravid silversides were collected by seine net from southern Rhode Island, USA, estuaries and returned to the laboratory. On each occasion, eggs were stripped from at least 10 females and fertilized by sperm from at least 20 males. The fertilized eggs were then incubated on nylon screens according to the methods of Barkman and Beck (1976). Offspring from these two artificial spawnings are referred to below as batch 1 and batch 2. After hatching, the larvae from each batch were divided into two groups and reared in 0.5 m diameter plastic containers with flow-through filtered seawater at 23 ± 2°C. Fish in one container were fed Reference Artemia, those in the other container San Pablo Bay Artemia. After 22 days of rearing, a portion of the batch 1 fish were collected from each and used in a 48-h toxicity test. After 25 days of rearing, a second portion of the batch 1 fish were collected from each container and used in a 96-h toxicity test. The batch 2 fish were reared for 26 days before a portion of the fish were collected from each container and used to repeat the 96-h toxicity test.

Sheepshead minnow - Fertilized eggs were obtained over a 24-h period from a laboratory population of adults held in a screened spawning chamber. The eggs were divided into two groups and incubated in two 18 cm diameter glass culture dishes containing filtered seawater. Upon hatching, the larvae remained separated in two groups and were transferred to 37-l aquaria with

undergravel filters and kept at 23± 1°C. One group was fed Reference Artemia, the other group San Pablo Bay Artemia, for 21 days, after which time the fish were used in a 96-h toxicity test.

Toxicity Testing

All the toxicity tests were conducted in flow-through glass exposure chambers, which measured 38 x 32 x 16 cm for the C. variegatus test with endosulfan and 41 x 16 x 15 cm for the M. bahia and M. menidia tests with cadmium. In both cases the system used was a siphon-flush mechanism, as described in Sosnowski et al. (1979). Delivery of toxicant solutions to the chambers was through a serial diluter (Benoit et al., 1982) in the case of cadmium tests, or a proportional diluter (Mount and Brungs, 1967) in the case of the endosulfan tests. The system used for the cadmium tests produced a 50% volume exchange in each experimental chamber every 3 hours, whereas the system used for the endosulfan test produced a 50% volume exchange every 4 hours.

Within each exposure chamber, test animals were held in four cylindrical cups (9 cm diameter, 10 cm high) with bottoms made of glass and sides made of 250 μm mesh nylon screen. Two of the cups in each chamber contained animals that were fed Reference Artemia nauplii and the other two cups contained animals that were fed San Pablo Bay Artemia nauplii. Preliminary testing had shown that Artemia nauplii could not pass from cup to cup. In each toxicity test there were duplicate exposure chambers for each toxicant concentration and for each control. Exposure chambers were arranged randomly. At the start of each test, the experimental animals were randomly distributed into the cups for the appropriate Artemia type. The mysid experiments began with 7 organisms per cup, whereas the silverside and sheepshead minnow experiments began with 10 organisms per cup.

Acute (48-h and 96-h) toxicity tests were conducted according to ASTM Standard Methods (ASTM, 1980). In the M. bahia chronic test, males and females at sexual maturity (on day 12) were redistributed to provide a more equal male:female ratio in each cup. Redistribution was only effected between replicate cups of the same Artemia type within a single exposure chamber. After the mysids began reproducing, newly-hatched juvenile M. bahia were counted and removed from each cup on the day that they appeared. At the end of the experiment, the total number of progeny produced per female was calculated for each concentration, based on total number of juvenile M. bahia removed divided by the number of females found in that concentration during the redistribution process.

All the tests were conducted under a 12L:12D light regime. Temperature and salinity conditions for each test are given in Table 1.

Calculation of LC50 values obtained from the toxicity tests was accomplished using either probit analysis (Finney, 1971) or moving average method (Kendall and Stuart, 1973), depending on which test's assumptions were met by the data (Stephan, 1977). Statistical comparison of LC50 values for Reference-fed organisms and San Pablo Bay-fed organisms was often impossible because of different methods of calculation of the values. Therefore, the LC50 values were condsidered to be significantly different only if their 95% confidence limits did not overlap.

Chemical analysis

Endosulfan - From each exposure chamber, 800 ml of seawater was collected and shaken in a separatory funnel with 80 ml of dichloromethane (DCM). The DCM layer was drained into a brown bottle with Teflon-lined cap and stored at room temperature until analyzed. The contents of the bottle were decanted into a 100 ml graduated cylinder, volume was recorded, and the sample was

then transferred to a Kuderna-Danish apparatus and concentrated to near dryness. Several small portions of hexane were added and re-evaporated to insure removal of all DCM. Solutions were diluted with hexane to give final concentrations of 20 to 200 ng/ml for injection into a Tracor MT-220 gas chromatograph equipped with a 1.83 m glass column (containing 4% SE-30/6% QF-1) and linearized electron capture detectors. Quantitation was by peak heights vs. standards of endosulfan I and endosulfan II, assuming the volume of DCM recovered was an aliquot of the 80 ml of DCM originally equilibrated with the seawater.

Cadmium - Duplicate samples were pipetted from each exposure chamber and acidified to 10% acid with concentrated nitric acid (J.T. Baker "Ultrex"). In each case the polyethylene vials and bottles were precleaned in 10% nitric acid and rinsed with deionized water.

Three atomic absorption instruments were used for analysis. The Perkin-Elmer model #603 with deuterium arc background correction was used for flame atomization analysis of all samples above 500 ppb Cd. Both the Perkin-Elmer model #5000 with Zeeman background correction and the Perkin-Elmer model #403 with deuterium arc were used for graphite furnace atomization of samples below 500 ppb Cd. The instrument set-up procedures were in accordance with standard procedures (EPA, 1979) and also in manufacturers' reference manuals.

The absorbance values from the Perkin-Elmer model #5000 were automatically sent to the Perkin-Elmer data station model #3600 and the other results were entered manually. Absorbance values were converted by means of non-linear regression analysis into concentration values. Methods such as replicate analysis, blank analysis and matrix matching for salt and acid content were used to correct for chemical interference and instrumental

drift. The analytical blank values determined were less than 2% of the average values determined and were insignificant. At cadmium concentration of 10 ppb, the signal could be reproduced to a precison of 5% or better.

RESULTS

The results show that feeding different geographical strains of brine shrimp to marine organisms can significantly alter the outcome of toxicity tests. Because the effects vary with species, the results are given separately for each species below.

Mysid shrimp - The mysids were cultured separately for three generations on Reference Artemia and on San Pablo Bay Artemia with the original intent that offspring (newly-released juveniles) from the third generation would be used in an acute toxicity test. After 70 days, however, an examination of the adult mysids showed that, whereas the Reference-fed (RAC-fed) mysids were carrying normal young in their brood pouches, the San Pablo Bay-fed (SPB-fed) mysids were carrying no young at all. The SPB-fed mysids had effectively stopped reproducing. This demonstrated the importance of adequate nutrition even in the production of animals intended for use in toxicity tests.

Adult mysids taken from the 70-day-old cultures were used in an acute toxicity test and showed significant differences in 48-h LC_{50} values (Table 2). The LC_{50} value for RAC-fed mysids was about 1.5 times that of the SPB-fed mysids. Furthermore, there was no overlap of the 95% confidence limits around the two LC_{50} values.

Newly-hatched juvenile mysids obtained from RAC-fed adults gave very different results when fed either RAC or SPB Artemia during a 28-day full-life-cycle chronic toxicity test (Table 3). The RAC-fed-juveniles exhibited

good control survival, a good dose-response relationship, and good reproduction upon reaching adulthood. In contrast, the SPB-fed juveniles showed poor control survival, no dose-response relationship (all treatments, including controls, gave less than 50% survival), and almost total lack of reproduction at adulthood. Mysids fed on RAC began producing young on day 18, but SPB-fed mysids produced offspring only on the last day (28) of the experiment. The results for the RAC-fed mysids were similar to those of Gentile et al. (1982), but an LC_{50} value could not be calculated for the SPB-fed mysids because survival in each treatment was less than 50%. Most mortality of SPB-fed mysids occurred during the second week of the experiment, concurrently with the onset of sexual maturity. It appears that mortality reflected the poor quality of the diet, rather than a toxicant effect, and that the diet effect was strongest at a time of major change (sexual maturity) in the organism's life cycle.

Atlantic silverside - In three acute toxicity tests, RAC-fed silversides consistently exhibited significantly higher 48-h and 96-h LC_{50} values than SPB-fed silversides (Table 4). In experiment 1, the 48-h LC_{50} was about four times higher; in experiments 2 and 3, the 96-h LC_{50}'s were almost three times higher. There was no overlap of confidence limits in any of the experiments. Survival of controls exceeded 90% in all cases, except for the SPB-fed fish in experiment 3 (85% survival).

Sheepshead minnow - In an acute toxicity test, the 96-h LC_{50} value (Table 5) for RAC-fed minnows was almost 20% higher than that for SPB-fed minnows; however, the 95% confidence limits for the two groups overlapped. Survival was significantly different for the two groups in the median concentration treatment (x^2 test; $p < 0.10$).

DISCUSSION

These experiments demonstrate serious problems that can result from use of brine shrimp nauplii from different geographical strains as a diet for test animals. First, a toxicologist could use valuable resources of time and money to culture organisms in the laboratory, only to find that the organisms were unable to reproduce the offspring needed for a toxicity test. Second, if the offspring were obtained, use of nutritionally poor brine shrimp nauplii during the test could cause the test results to be invalid or unacceptable because of high mortality in the control. That problem is especially costly during chronic tests and can be perplexing because the investigator may not understand the cause of the problem. Third, investigators could conduct tests and obtain apparently valid results, yet those results could vary by as much as 2-4 times.

Our findings are significant because of the widespread use of Artemia nauplii as a food for larval fish and crustaceans prior to and/or during marine toxicological testing. The marine ecotoxicologist can choose to buy brine shrimp cysts (from which to hatch the nauplii) from any of at least eight commercial suppliers who collect their products from at least eight different geographical sources (Sorgeloos, 1981). Our reasons for choosing the two strains used in this study were that one (RAC) was specifically available as a previously tested, high quality standard against which to test all other strains and the other (SPB) was a commercially available product that had yielded mixed results when used in culture tests on a variety of marine species. Johns et al. (1980), Beck and Bengtson (1982), Johns et al. (1981), and Usher and Bengtson (1981) found, respectively, that SPB Artemia caused complete mortality of crab larvae, about 43% mortality of Atlantic silverside larvae, about 20% mortality of mysid

shrimp juveniles, and no mortality of sheepshead minnow larvae. Our results reflect those findings in that sheepshead minnow larvae exhibited the smallest difference in LC_{50} values between SPB-fed and RAC-fed populations, whereas mysids shrimp and Atlantic silversides exhibited larger, significant differences during the acute tests.

Most mortality of Atlantic silversides fed SPB Artemia occurs during the first two weeks after hatching in normal culture conditions (Beck and Bengtson, 1982). Thus, during the last 7-10 days prior to toxicity testing in this study, survival was good and met the ASTM standard (ASTM, 1980) of less than 3% mortality of stock fish during the 48h immediately preceding the test. On the other hand, use of SPB-fed Atlantic silversides in a 28-day early life-stage test would undoubtedly cause invalidation of the test results due to excessive mortality of control fish in the first two weeks of life. The excessive mortality of SPB-fed mysid shrimp in the 28-day test reported here was predictable from the results of Johns et al. (1981), who found 57% mortality of mysids fed SPB for 25 days.

Previous experiments on diet effects in toxicity testing have used qualitatively very different diets, e.g., algae vs. trout granules (Winner et al., 1977) or trout pellets vs. tubificid worms (Phillips and Buhler, 1979). This is the first report of significantly altered toxicity test results due to intra-specific (geographical) differences in a single species of food organism. The results emphasize the need for assurance of the quality of diets that are fed to experimental organisms. Furthermore, they suggest the need for standardization of nutrition of organisms used in toxicity tests if the results of those tests are to be compared with results obtained in other laboratories.

In the United States, standardization of nutrition in aquatic toxicity testing is being implemented in at least two ways. First, the U.S. Environmental Protection Agency's Office of Toxic Substances is funding research to standardize the diets used by EPA and its contractors. So far, a new, homogeneous collection of brine shrimp cysts (Reference Artemia Cysts II) has been purchased and tested for use with marine organisms. Research on prepared (synthetic) diets and production of brine shrimp cysts in standard laboratory conditions will eventually allow EPA to obtain truly standard diets on a continual basis. Second, a Task Group on Brine Shrimp has been formed within the committee structure of ASTM to write standards for the use of Artemia in toxicity testing. Those standards will serve as a guideline for both government and private or industrial laboratories engaged in toxicity testing.

Diets provided to experimental organisms should ideally be similar, in terms of nutritional biochemistry, to their diets in the natural environment so that the laboratory organisms will be physiologically similar to their field counterparts. Physiological similarity, if considered at all in a given toxicity test, is usually estimated simply from growth and survival of the control organisms. Standardization of diets should have as its goal the assurance that physiological similarity exists not only among organisms in independent toxicity tests, but between those organisms and field organisms for extrapolation of data from the laboratory to the natural environment.

CONCLUSIONS

The diet fed to test species used in toxicological studies affects the quality of the data produced. Standard diets are required to minimize the

nutritional variable in such studies. The use of RAC's or other established standards (when available) is recommended when Artemia nauplii are required to feed species for toxicity testing.

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Table 1. Temperature and salinity conditions used in the toxicity tests described in this study.

Species	Test Duration	Temperature (°C)	Salinity (°/oo)
M. bahia	48-h	25 ± 1	29 ± 1
M. bahia	28-days	25 ± 1	28 ± 2
M. menidia	48-h (expt. 1)	22 ± 1	29 ± 1
M. menidia	96-h (expt. 2)	22 ± 1	29 ± 1
M. menidia	96-h (expt. 3)	22 ± 1	31 ± 1
C. variegatus	96-h	23 ± 1	31 ± 1

Table 2. Percent survival of adult mysids shrimp (*Mysidopsis bahia*) in a 48-h toxicity test with cadmium, fed either Reference *Artemia* (RAC) or San Pablo Bay *Artemia* (SPB) prior to and during the test.

Cadmium Concentration (ppb)		% Survival	
Nominal	Measured	RAC	SPB
0	0.0	96	89
25	31.9	96	79
50	62.5	68	48
100	108.7	51	3
200	207.9	0	0
400	463.2	0	0
	48-h LC_{50} value (ppb)	98.5	64.4
	95% confidence limits (ppb)	85.3 - 113.8	55.9 - 71.9

Table 3. Weekly percentage survival of mysids in a 28-day life-cycle toxicity test. Columns labelled RAC indicate mysids fed Reference *Artemia* during the test, those labelled SPB indicate mysids fed San Pablo Bay *Artemia* during the test. Average number of progeny produced per female (NPF) is also given as a measure of reproduction. Measured cadmium concentrations are given as mean ± standard deviation for 10 samples taken during the experiment.

Cadmium Concentration (ppb)		RAC % survival / week					SPB % survival / week				
Nominal	Measured	1	2	3	4	NPF	1	2	3	4	NPF
0.00	0.12 ± 0.10	100	93	93	82	2.0	71	18	18	14	0.0
1.25	1.37 ± 0.31	100	100	96	93	1.7	96	57	54	46	0.4
2.50	2.79 ± 0.40	93	82	75	71	6.9	82	43	39	39	0.0
5.00	5.36 ± 0.73	89	86	86	79	2.5	68	39	36	36	0.0
10.00	9.61 ± 1.61	88	68	52	20	1.2	82	32	21	14	0.0
20.00	19.50 ± 3.02	93	43	4	0	0	89	11	0	0	0.0
28-day LC_{50} value		5.6 ppb					(not calculable)				
95% confidence limits		4.6 - 6.9 ppb									

Table 4. Percent survival of Atlantic silversides (Menidia menidia) in acute toxicity tests with cadmium fed either Reference Artemia (RAC) or San Pablo Bay Artemia (SPB) prior to and during the tests. Experiment 1 lasted 48 h, experiment 2 and 3 each lasted 96 h. Measured concentrations are given as mean ± standard deviation.

EXPERIMENT 1

Cadmium Concentration (ppb)		% Survival	
Nominal	Measured	RAC	SPB
0	7 ± 0	100.0	100.0
625	933 ± 4	72.5	25.0
1250	1535 ± 41	62.5	10.0
2500	2878 ± 224	27.5	10.0
5000	5130 ± 24	5.0	0.0
10,000	7443 ± 226	7.5	0.0
	48-h LC_{50} value (ppb)	1731	439
	95% confidence limits (ppb)	1396 - 2069	124 - 716

EXPERIMENT 2

Cadmium Concentration (ppb)		% Survival	
Nominal	Measured	RAC	SPB
0	5 ± 2	100.0	100.0
156	211 ± 2	100.0	82.5
313	343 ± 7	92.5	35.0
625	622 ± 12	67.5	2.5
1250	1197 ± 16	20.0	0.0
2500	2141 ± 27	7.5	0.0
	96-h LC_{50} value (ppb)	821	298
	95% confidence limits (ppb)	716 - 946	266 - 322

EXPERIMENT 3

Cadmium Concentration (ppb)		% Survival	
Nominal	Measured	RAC	SPB
0	5 ± 11	97.5	85.0
156	149 ± 19	90.0	77.5
313	314 ± 31	85.0	80.0
625	595 ± 82	80.0	40.0
1250	1199 ± 232	37.5	15.0
2500	1961 ± 164	15.0	7.5
	96-h LC_{50} value (ppb)	900	358
	95% confidence limits (ppb)	727 - 1166	253 - 490

Table 5. Percent survival of sheepshead minnows (Cyprinodon variegatus) in a 96-h acute toxicity test with endosulfan, fed either Reference Artemia (RAC) or San Pablo Bay Artemia (SPB) prior to and during the test.

Endosulfan Concentration (ppb)		% Survival	
Nominal	Measured	RAC	SPB
0.0[a]	0.00	100.0	100.0
0.0[b]	0.00	100.0	100.0
0.5	0.27	100.0	100.0
1.0	0.55	100.0	95.0
2.0	0.83	93.3	81.7
4.0	1.84	5.0	0.0
8.0	3.91	0.0	0.0
	96-h LC_{50} (ppb)	1.21	1.02
	95% confidence limits (ppb)	1.10 - 1.36	0.93 - 1.15

[a] control

[b] solvent control